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Supplemental Information

The Circadian Protein Nocturnin Regulates Metabolic Adaptation in Brown Adipose Tissue

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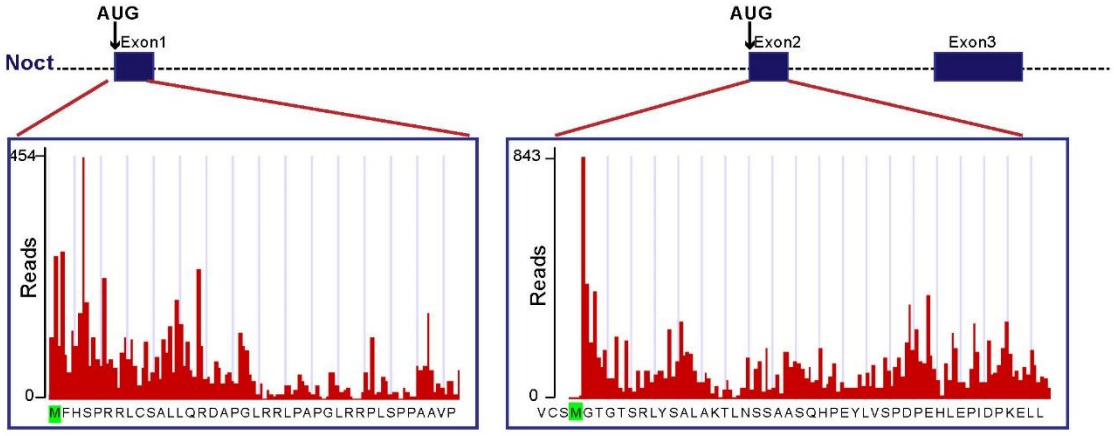
A.



B.

Ensembl Primary Identifier	Gene Symbol	Description	Organism	Mito Targeting Seq iPSORT	Mito Targeting Seq TargetP	Mito Targeting Seq MitoProt	Mito Targeting Seq MitoFates
ENSG00000151014	NOCT	nocturnin	H.sapiens	1	0.923	0.9864	0.942
ENSMUSG00000023087	Noct	Nocturnin	M. musculus	1	0.873	0.9821	0.733

C.

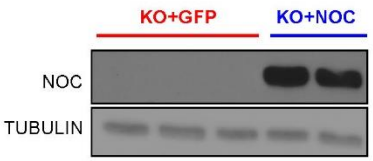


Figure S1, related to Figure 1.

(A) Global aggregate for elongating ribosome profiles from all studies in human cell lines, reproduced from GWIPS-viz database (Michel et al., 2014). (B) Mitochondrial targeting sequence predictions reproduced from MitoMiner 4.0 database (Smith and Robinson, 2016). (C) Confirmation of stable Nocturnin expression in the *Nocturnin*$^{-/-}$ MEFs expressing rescue constructs as measured by western blot.

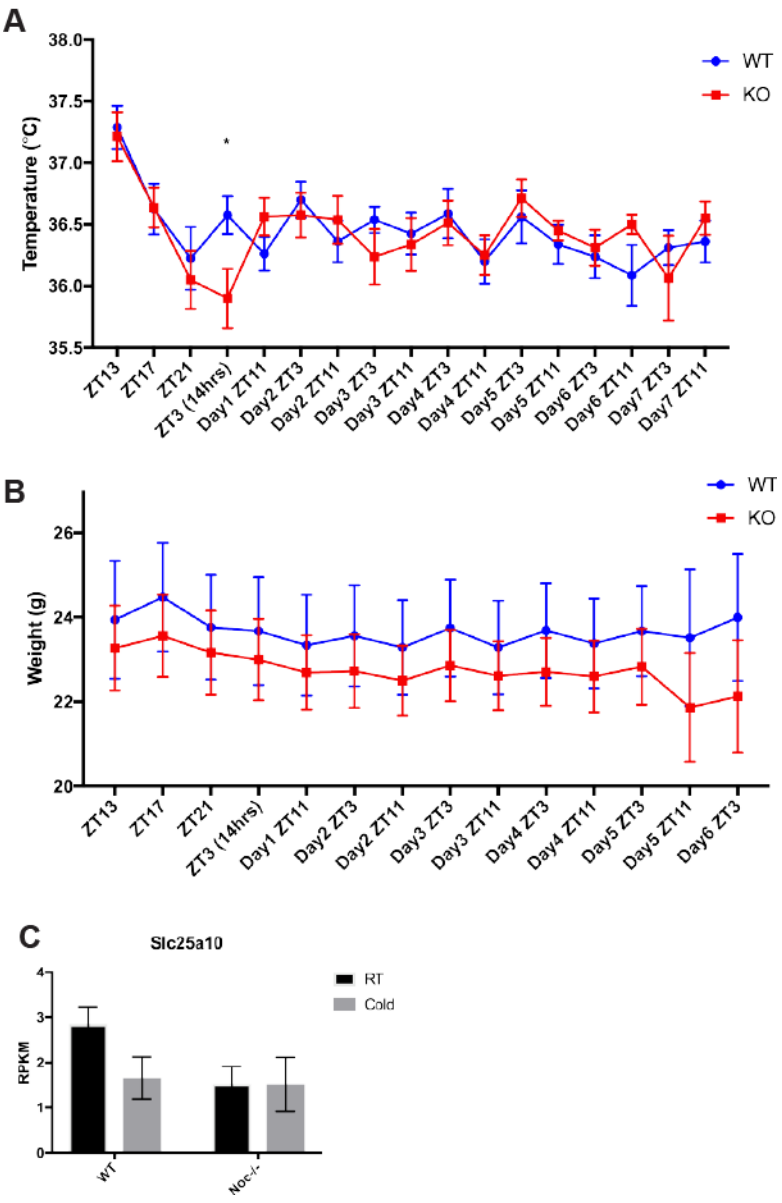


Figure S2, related to Figure 4

(A) Bi-daily (ZT3, ZT11) measurements of core body temperature in wild-type and *Nocturnin*$^{-/-}$ mice kept at 6°C for 7 days. (B) Bi-daily (ZT3, ZT11) measurements of bodyweight of wild-type and *Nocturnin*$^{-/-}$ mice kept at 6°C for 7 days. (C) RPKM values for Slc25a10 gene expression in *Nocturnin*$^{-/-}$ and WT mice BAT kept at RT or cold conditions for 4hrs. Data represent mean +/- s.e.m.

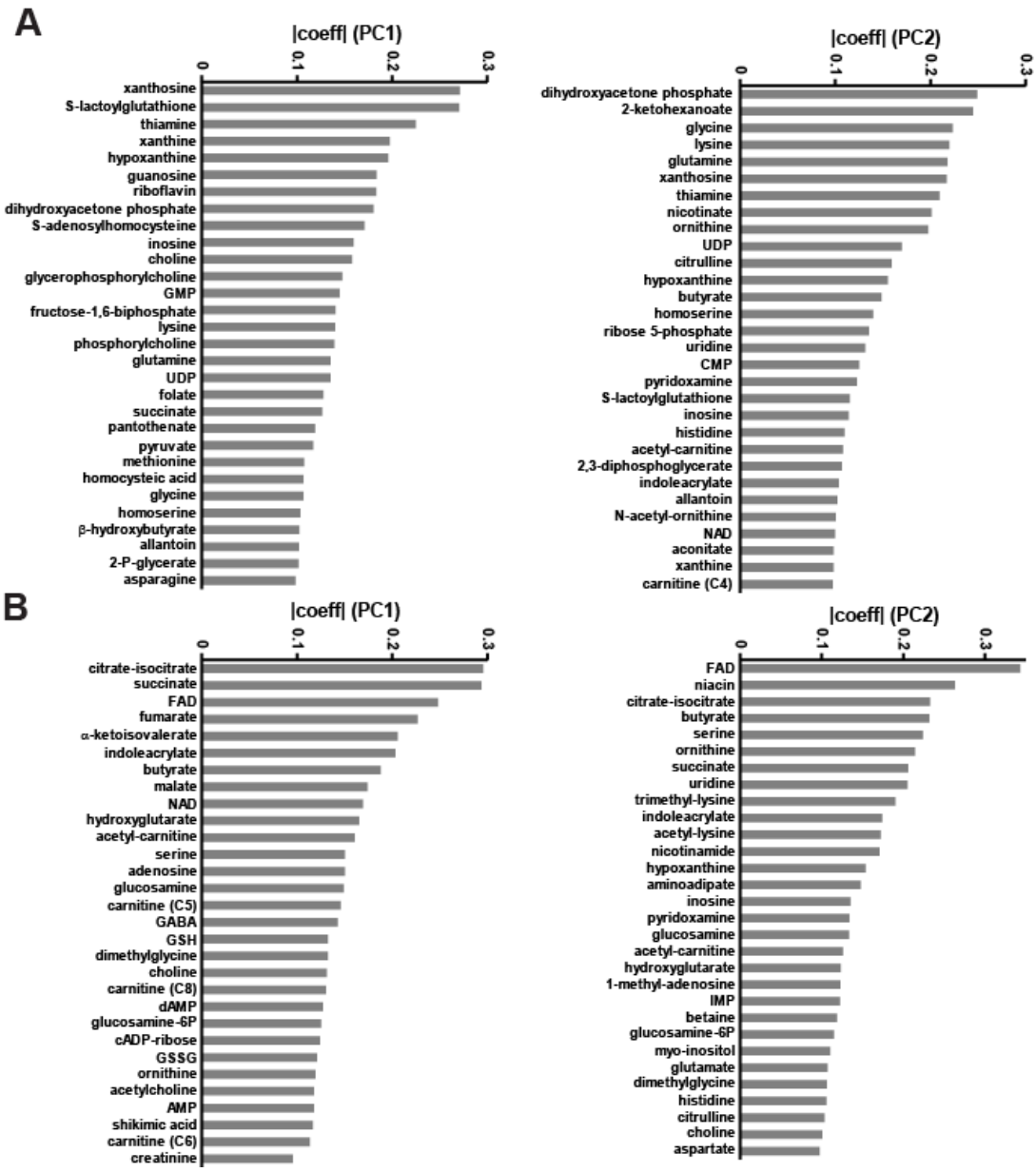


Figure S3, related to Figure 4

(A) Contribution of the top 30 metabolites contributing to principal components 1 (PC1) and 2 (PC2) for the principal component analysis of 4hr metabolomics shown in Fig. 4B. Absolute magnitude of coefficients for each metabolite for PC1 or PC2 are plotted. (B) Contribution of the top 30 metabolites contributing to PC1 and PC2 for the principal component analysis of 7 day metabolomics shown in Fig. 4D. Absolute magnitude of coefficients for each metabolite for PC1 or PC2 are plotted.

TRANSPARENT METHODS

Animal Studies

Male and female wild-type and *Nocturnin*$^{-/-}$ C57BL/6 (congenic, N>9) mice were group-housed, under standard laboratory conditions, including a 12-hr light/dark cycle and free access to regular chow (Harlan Teklad diet 2918) and water. For cold exposure experiments, age-matched 10-20 week old young adult male and female WT and *Nocturnin*$^{-/-}$ mice were randomly assigned to cold (6°C) or room temperature (22°C) groups. Mice were single housed for the duration (4hrs) of the experiment and food was removed in order to minimize individual variation. For the 7-day cold exposure protocol, WT and *Nocturnin*$^{-/-}$ mice were single housed at 6°C for the duration of the experiment with free access to food and water. At the end of the experiment, mice were killed by decapitation and BAT was dissected and immediately frozen in liquid nitrogen and stored at -80°C. For the metabolomics analysis, tissue from male mice were used only. All animal studies were conducted in accordance with IACUC regulations and guidelines.

Plasmids and Cell lines

To generate the Nocturnin-FLAG lentivirus construct, the mouse Nocturnin cDNA with 3x flag tag was subcloned into the Age1 and EcorR1 sites of pLJM1-EGFP. pLJM1-EGFP plasmid was obtained from Addgene (Plasmid#19319). Single point mutants were made using Q5 Site-Directed Mutagenesis Kit according to manufacturer's instructions by some modifications. Briefly exponential amplification was performed with PCR using the primers with the point mutations mNOC (M1A), mNOC (M65A) and the template plasmid (pLJM1-mNOC). Next day, Kinase, Ligase& Dpnl (KLD) treatment was performed with some modifications. Instead of using 10X KLD enzyme mix, 1μl Quick ligase, T4 PNK and Dpn1 were individually added to the mixture. Next, transformation was performed using DHSα competent cells according to manufacturer's instructions, using ampicillin selection plates. After overnight incubation at 37°C, colonies were

selected and bacterial cultures were grown with LB and the selection marker. Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA was then sent to sequencing for the confirmation of the point mutations.

Lentiviral particles were produced according to Addgene protocol. Briefly, pLJM1-EGFP, pLJM1-NOC, pLJM1-NOC(M1A) and pLJM1-NOC(M65A) plasmids were mixed with packaging and envelope plasmids and with FUGENE Transfection Reagent (Promega) in serum-free OPTI-MEM, so that FUGENE: total DNA ratio will be 3:1. 50-80% confluent HEK-293T cells (in DMEM that does not contain antibiotics) were then transfected with this mixture. Cells were incubated at 37°C, 5% CO_2 for 12-15 hours. Next morning, transfection reagent was removed and replaced with fresh media (DMEM+10%FBS+penicillin/strepromycin). Cells were incubated for another 24 hrs at 37°C, 5% CO_2. On Days 4 and 5, lentiviral particles were collected and stored at -80°C until use.

Generating Stable Cell Lines

Immortalized mouse embryonic fibroblast (MEF) cell lines were maintained in high-glucose DMEM supplemented with 100U/ml penicillin, 10μg/ml streptomycin and 10% fetal bovine serum. To generate stable cell lines, 70% confluent MEF cells were infected with 0.1-1mL lentiviral particles. Polybrene (8μg/ml) was added to the media to increase viral infection efficacy. Titration was performed in order to assess the infection efficacy and to decide the optimal concentration of lentiviral particles for the desired gene expression level. Cells were incubated overnight and changed to fresh media after 24 hrs and selected with puromycin. Lentiviral infection was visually confirmed for the pLJM1-GFP construct and western blotting was performed to confirm the expression of other target genes.

Temperature Measurements

Wild-type and *Nocturnin*$^{-/-}$ mice were gender-matched so that there are equal number of male and female for each genotype. Mice were single housed at 6°C with free access to food and water for the duration of the experiment and core body temperature was measured by a rectal thermometer every 4 hours (first day) and 12 hours (daily for the following 6 days). Body weight measurements were taken for mice at these same timepoints.

qRT-PCR

Total RNA was extracted from BAT tissue by Trizol (Invitrogen) using a dounce homogenizer (Wheaton). 1ug of total RNA was used to make complementary DNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Diluted cDNAs (1/100) were amplified by Power SYBR Green Master Mix (Applied Biosystems) using gene specific primers. Relative mRNA levels were calculated by quantitative PCR method and normalized to B2M gene. The following primer pairs were utilized:

Gene	Forward Primer	Reverse primer
MT-ND1(Mouse)	AATCGCCATAGCCTTCCTAA	GCGTCTGCAAATGGTTGTAA
MT-ND2 (Mouse)	CACAATATCCAGCACCAACC	GAGGCTGTTGCTTGTGTGAC
MT-CO1 (Mouse)	GGTGGTCTAACCGGAATTGT	GATAGCAAACACTGCTCCCA
MT-CO2 (Mouse)	GCCGACTAAATCAAGCAACA	CAATGGGCATAAAGCTATGG
MT-ATP8 (Mouse)	GGCACCTTCACCAAAATCACT	TGGGGTAATGAATGAGGCAAAT
MT-ATP6 (Mouse)	CAGGCTTCCGACACAAACTA	TGTAAGCCGGACTGCTAATG
MT-CO3 (Mouse)	GCCCTCCTTCTAACATCAGG	GCCTTGGTAGGTTCCTTCAC
MT-ND3 (Mouse)	AATGCGGATTCGACCCTA	TGAATTGCTCATGGTAGTGGA
MT-ND4L (Mouse)	ACTCCAACTCCATAAGCTCCA	TTTGGACGTAATCTGTTCCG
MT-ND4 (Mouse)	TATTACCCGATGAGGGAACC	AGGGCAATTAGCAGTGGAAT
MT-ND5 (Mouse)	ACCCATGACTACCATCAGCA	GGAATCGGACCAGTAGGAAA
MT-ND6 (Mouse)	AGCACAACTATATATTGCCGCTAC	GATGGTTTGGGAGATTGGTT
MT-CYTB (Mouse)	CATTCTGAGGTGCCACAGTT	GATGAAGTGGAAAGCGAAGA
MT-RNR2 (Mouse)	CCTAGGGATAACAGCGCAAT	ATCGTTGAACAAACGAACCA
MT-RNR1 (Mouse)	TGAGCAATGAAGTACGCACA	TTCCAAGCACACTTTCCAGT
mtDNA (Mouse)	CCTATCACCCTTGCCATCAT	GAGGCTGTTGCTTGTGTGAC
nDNA (Mouse)	ATGGAAAGCCTGCCATCATG	TCCTTGTTGTTCAGCATCAC
Noct-Exon3 (Mouse)	ACCAGCCAGACATACTGTGC	CTTGGGGAAAAACGTGCCT
Ucp1 (Mouse)	GGCCTCTACGACTCAGTCCA	TAAGCCGGCTGAGATCTTGT
Pparg (Mouse)	AGGCGAGGGCGATCTTGACAG	AATTCGGATGGCCACCTCTTTG
Ppargc1a (Mouse)	GAAGAGATAAAGTTGTTGGTTTGGC	AGACAAATGTGCTTCGAAAAAGAA
B2m (Mouse)	CTCGGTGACCCTGGTCTTC	TTGAGGGGTTTTCTGGATAGCA
Dio2 (Mouse)	CAGTGTGGTGCACGTCTCCAATC	TGAACCAAAGTTGACCACCAG

RNA-seq analysis

mRNA-seq libraries were prepared as previously described (Takahashi et al., 2015). Sequencing was performed by Nextseq Sequencing System (Illumina) on individually barcoded samples. Average sequencing depth was 55 million reads/sample. Using fastqc, the reads were checked for quality followed by treatment with an in-house python script to trim low quality reads. Reads were aligned with STAR (Dobin et al., 2013) against mouse genome (mm10) and gene annotation Mv14 from Gencode (Frankish et al., 2018), with parameters "--readFilesIn input.trim.gz --readFilesCommand zcat --sjdbGTFfile M14.annotation.gtf --outFilterType BySJout --outFilterMultimapNmax 10 --alignSJoverhangMin 10 --alignSJDBoverhangMin 1 --outSAMtype BAM SortedByCoordinate --outSAMunmapped Within --outFilterMismatchNmax 3 --twopassMode Basic". BAM file was filtered for ribosomal RNA and secondary alignments by keeping the uniquely mapped reads. Coverage tracks were generated using in-house python script by normalizing each sample to 10million total reads. HTSeq-count was used for raw read counting (Anders et al., 2015) and RPKM was calculated using a custom R script.

Differential gene expression analysis

The raw read counts from HTSeq-count were filtered for genes not expressed. Filtered counts were used to identify differentially expressed genes using DESeq2 (Love et al., 2014). Genes with FDR < 0.05 and |log2(FC)| > 0.3 were considered differentially expressed. Gene ontology analysis was performed using GOstat package in R (Falcon and Gentleman, 2007) and further confirmed with ToppGene Suite (Chen et al., 2009). Gene Ontology's categories were considered with FDR < 0.05 and number of genes per category > 5.

Co-expression network analysis

To identify modules of co-expressed genes in the RNA-seq data, we carried out weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008). A soft-threshold

power was automatically calculated to achieve approximate scale-free topology (R^2>0.85, Power = 12). Networks were constructed with blockwiseModules function with biweight midcorrelation (bicor). We used corType = bicor, maxBlockSize = 16000, mergingThresh = 0.15, reassignThreshold = 1e-6, deepSplit = 4, detectCutHeight = 0.999, minModuleSize = 30, networkType=signed. The modules were then determined using the dynamic tree-cutting algorithm. To ensure robustness of the observed network, we used a permutation approach recalculating the networks 200 times and comparing the observed connectivity per gene with the randomized one. None of the randomized networks showed similar connectivity, providing robustness to the network inference. Module visualization was performed using Cytoscape (Shannon et al., 2003). Functional enrichment was performed using GOstat package in R (Falcon and Gentleman, 2007).

Immunocytochemistry and Confocal Microscopy

Standard immunocytochemistry protocols were used. Briefly, cells were washed twice with PBS, incubated with 300nM MitoTracker Red CMXRos for 30 minutes at 37°C prior to fixation (4%PFA, 37°C, 10 minutes). Cells were then washed 3 times with PBS, permeabilized (0.2% TritonX/PBS, 10 minutes), blocked (3%BSA/PBS for 30 minutes) and incubated overnight with the primary antibody (αFLAG M2 (Sigma F1804)) added to the blocking solution. The next day, cells were washed 3 times with PBS and incubated with secondary antibody (AlexaFluor 488 (Life Technologies)), followed by DAPI (5 min). After another wash cells were mounted onto coverslips. Confocal images were acquired using a Zeiss LSM710 confocal microscope, and images were analyzed using Zen software.

Mitochondrial Respiration Assay

Cellular respiration was measured using the Seahorse XFe96 analyzer (Seahorse Biosciences, Agilent technologies). Briefly, 14,000 cells were plated into micro well plates the night before the assay. The next day, the cell culture media was replaced with assay media (DMEM (Sigma D5030) supplemented with 10% FBS, 1% penicillin/streptomycin, 2mM glutamine, 1mM pyruvate, 10mM glucose). Cells were equilibrated to the assay media for one hour (37°C) before analysis. The final concentrations for drugs used in the experiment were: oligomycin (2μM), CCCP (10μM) and antimycinA (2μM). Protein concentrations were calculated using a BCA assay kit (ThermoFisher), and used to normalize oxygen consumption measurements.

Mitochondrial respiration in permeabilized cells (10,000 cells / well) was measured using the XF PMP reagent (Agilent Technologies), according to manufacturer's instructions. Briefly, cells were exchanged into assay buffer (2mM HEPES, 220mm mannitol, 70mM sucrose, 10mM KH_2PO_4, 5mM $MgCl_2$, 1mM EGTA, pH7.4), supplemented with 2nM PMP reagent, and either complex I substrates (10mM pyruvate, 1mM malate) or complex II substrate (10mM succinate, 2μM rotenone). Oxygen consumption was measured under basal conditions and following injection of antimycin A (final concentration 2uM) in order to calculate state 4 (uncoupled) mitochondrial respiration.

DNA Extraction

Mitochondrial and genomic DNA was extracted from BAT tissue using DNeasy Blood and Tissue Kit (QIAGEN) according to manufacturer's instructions. 10ng of DNA was then amplified by qRT-PCR using primers for genomic and mitochondrial DNA.

Mitochondrial Fractionation and Proteinase K Assay

Mitochondrial isolation was performed as previously described (Frezza et al., 2007). Briefly, five confluent 10cm plates of HEK 293 cells were collected using a cell scraper, then resuspended in

3ml of isolation buffer (10mM HEPES, 220mM mannitol, 70mM sucrose, 5mM $MgCl_2$, 1mM EGTA, 20mM KCl, pH7.4). Approximately 1,000,000 cells were collected, spun down, and lysed in 100ul of RIPA buffer for the whole-cell lysate. The rest of the cell suspension was mechanically disrupted using a glass-Teflon dounce homogenizer. The lysates were spun down at 600g for 10 minutes at 4°C to pellet unbroken cells and nuclei. The supernatant was collected and centrifuged again at 7,000g for 10 minutes at 4°C to pellet the mitochondrial fraction. The mitochondrial pellet was washed with 200ul of isolation buffer and then equally split into 7 different microcentrifuge tubes. The pellet was spun down at 7,000g for 10 minutes at 4°C, and the supernatant was discarded. The mitochondrial pellets were resuspended in 40ul of reaction buffer. Briefly, 100μg/ml Proteinase K and/or 1%TritonX-100 were added to the samples in isolation buffer and samples were incubated on ice for 30 minutes. The reaction was stopped by the addition of 2ul of 100mM PMSF and 10ul of 5x SDS sample buffer. Ten microliters of each sample were loaded for SDS-PAGE and Western blot analysis, probing with antibodies raised against: Tubulin (Sigma T6199), Nocturnin (in house antibody ((Niu et al., 2011)), and MT-CO1 (Invitrogen 459600). Mitochondrial fractionation from BAT was similarly performed, and extracts were probed with antibodies against Nocturnin (in house antibody (Niu et al., 2011)), VDAC (Cell Signaling D73D12), MT-CO2 (Proteintech 55070-1-AP), and Actin (Millipore MAB1501).

BAT Metabolomics

Frozen BAT tissue (50-100mg) was homogenized in chloroform/methanol/H_2O (40:40:20), and metabolites were extracted from the polar layer. Samples were subjected to three freeze-thaw cycles, and spun at 14000rpm for 15min to pellet precipitants. The supernatant was transferred to a new tube and evaporated overnight. For LC-MS/MS measurements, dried metabolites were resuspended in 0.03% formic acid, vortexed and centrifuged to remove debris. Samples were randomized and blinded prior to analysis on an AB QTRAP 5500 liquid chromatograph/triple quadropole mass spectrometer (Applied Biosystem SCIEX). Chromatogram review and peak

area integration was performed in MultiQuant software. Peak areas were normalized against total ion count.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. Two-way analysis of variance (ANOVA) followed by *post hoc* tests or two-tailed Student's t-tests were performed as indicated. For metabolomics, hierarchical clustering was performed in Cluster 3.0, and principal component analysis was performed in MATLAB (MathWorks, Inc.).

Availability of data and material

The NCBI Gene Expression Omnibus (GEO) accession number for the data reported in this manuscript is **GSE133050 (token: yvmregeultsvtih).**

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